



Strong differences between two congeneric species in sensitivity to pesticides in a warming world

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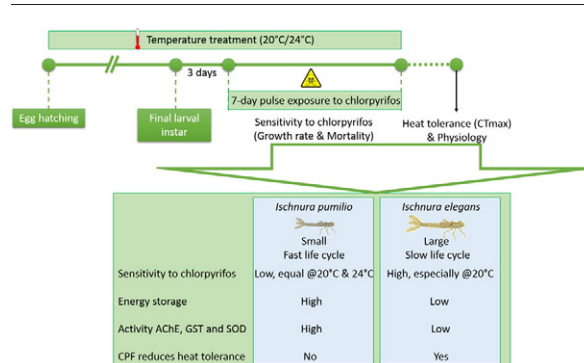
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HIGHLIGHTS

- Combined effects of warming and chlorpyrifos differed between two damselfly species.
- Chlorpyrifos reduced survival and growth more in the large, slow growing species.
- Chlorpyrifos reduced heat tolerance only in the more sensitive species.
- Differences in sensitivity were not driven by size and life history but physiology.
- Trait-based methods to predict sensitivity to pesticides need physiological traits

GRAPHICAL ABSTRACT



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ABSTRACT

To predict the impact of pesticides in a warming world we need to know how species differ in the interaction pathways between pesticides and warming. Trait-based approaches have been successful in identifying the 'pace of life' and body size as predictors of sensitivity to pesticides among distantly related species. However, it remains to be tested whether these traits allow predicting differences in sensitivity to pesticides between closely related species, and in the strength of the interaction pathways between pesticides and warming. We tested the effects of multiple pulses of chlorpyrifos (allowing accumulation) under warming on key life history traits, heat tolerance (CT_{max}) and physiology of two congeneric damselfly species: the fast-paced (fast growth and development, high metabolic rate), small *Ischnura pumilio* and the slow-paced, large *I. elegans*. Chlorpyrifos reduced survival and growth, but contrary to current trait-based predictions *I. pumilio* was 8× less sensitive than *I. elegans*. The lower sensitivity of *I. pumilio* could be explained by a higher fat content, and higher activities of acetylcholinesterase and of detoxifying and anti-oxidant enzymes. While for *I. pumilio* the effect of chlorpyrifos was small and did not depend on temperature, for *I. elegans* the impact was higher at 20 °C compared to 24 °C. This matches the higher pesticide accumulation in the water after multiple pulses at 20 °C than at 24 °C. The expected reduction in heat tolerance after pesticide exposure was present in *I. elegans* but not in *I. pumilio*. Our results demonstrate that closely related species can have very different sensitivities to a pesticide resulting in species-specific support for the "toxicant-induced climate change sensitivity" and the "climate-induced toxicant sensitivity" interaction pathways. Our results highlight that trait-based approaches can be strengthened by integrating physiological traits.

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1. Introduction

Pesticides and warming have the potential to interact, thereby challenging ecological risk assessment (ERA) of pesticides in a warming world (Noyes and Lema, 2015). On the one hand, the toxicity of many pesticides increases under warming (“climate-induced toxicant sensitivity”, Noyes and Lema, 2015). On the other hand, the ability to cope with high temperatures decreases under pesticide exposure (“toxicant-induced climate change sensitivity”, Hooper et al., 2013, Moe et al., 2013, Noyes and Lema, 2015). While both interaction pathways have large support, studies focused almost exclusively on the sensitivities of single species (but see Boeckman and Bidwell, 2006, Patra et al., 2007). To advance ERA in a warming world we need to know whether species predictably differ in these interaction pathways.

In general, ERA uses two different approaches to extrapolate sensitivity estimates from tested species to species with unknown sensitivity. In phylogeny-based approaches, the phylogenetic relatedness is used to infer similarity in sensitivity. The underlying assumption is that more related species have a more similar sensitivity (Hammond et al., 2012). In trait-based approaches, selected species traits are used as predictors to infer species sensitivity (Rubach et al., 2011; Van den Brink et al., 2011; Wiberg-Larsen et al., 2016). Life history traits, particularly the ‘pace of life’ and body size, have been put forward as important predictor traits. Species with a faster ‘pace of life’ have a faster growth and development, and a higher metabolic rate and are therefore expected to be more sensitive to stressors (Réale et al., 2010). This is expected for several reasons. Species that invest more energy in growth and development are expected to invest less in processes such as detoxification and damage repair because of energy-mediated trade-offs (Congdon et al., 2001). Moreover, species with a higher resting metabolic rate have been shown to be more sensitive to pesticides because of the associated higher turnover rate of pesticides, by which the toxic compounds can more easily interfere and cause damage (Baas and Kooijman, 2015). Further, smaller animals with a larger surface area to volume ratio have a higher uptake of pesticides leading to an increased sensitivity (Buchwalter et al., 2002; Rubach et al., 2012). Trait-based approaches have been successful in identifying the ‘pace of life’ and body size as predictors of sensitivity to pesticides among distantly related species (Rubach et al., 2012; Baas and Kooijman, 2015). However, it remains to be tested whether this trait-based extrapolation is also valid to predict differences in sensitivity between closely related species.

While at higher temperatures many pesticides (like organophosphates) become more toxic (Noyes et al., 2009), the extent to which the total impact of these pesticides will change under warming will depend on the relative changes in toxicity and exposure. The most commonly used method in ecotoxicology testing is static renewal, where the pesticide medium is daily refreshed. In these static renewal studies the sensitivity to pesticides typically increases with higher temperatures (e.g. Sokolova and Lannig, 2008; Dinh Van et al., 2014; Noyes and Lema, 2015). But under warming higher pesticide degradation rates may reduce exposure (Hooper et al., 2013), assuming that pesticide input remains constant (but see Kattwinkel et al., 2011). The higher degradation rates under warming can counter the increased toxicity of organophosphate pesticides under warming, potentially leading to a lower impact of pesticides at higher temperatures (Op de Beeck et al., 2017a, 2017b). Under realistic multiple pulse scenarios where pollutants can accumulate (Reinert et al., 2002) and that are predicted to become more frequent under warming (Kattwinkel et al., 2011), degradation rates will become especially important. Multiple pulse scenarios are therefore important to consider to arrive at a more realistic ERA of pesticides in a warming world. Yet, studies testing both interaction pathways between pesticides and warming using multiple pulse scenarios are rare (but see Op de Beeck et al., 2017b).

The main objectives of the current study were (i) to test how warming influenced the impact of multiple pulses of a pesticide, (ii) to investigate how these pesticide effects influenced the heat tolerance,

and (iii) to test whether sensitivity and the strength of both interaction pathways between warming and pesticides differed between closely related species with a different ‘pace of life’. To address these objectives, we focused on effects of warming and pesticide exposure on the damselfly *Ischnura pumilio* which has a fast ‘pace of life’ (rapid growth and development, high metabolic rate), and compared these effects with those on the closely related *I. elegans* which has a slow ‘pace of life’ (Debecker and Stoks, unpublished data). Based on the phylogeny-based approach we predict both species to show a similar sensitivity to the pesticide, yet based on the trait-based approach we expect the faster-living and smaller *I. pumilio* to be more sensitive to the pesticide than the slower-living and larger *I. elegans* (based on Réale et al., 2010, Rubach et al., 2012, Baas and Kooijman, 2015). To allow direct comparison, populations of both species were collected at the same latitude in Europe and tested simultaneously in the laboratory using a common garden experiment from the egg stage. The results for the *I. elegans* populations have been included in two previous papers where we focused on the comparison of pesticide effects under warming between *I. elegans* populations from different latitudes in Europe (Op de Beeck et al., 2017a, 2017b); these data here serve as direct comparison for the populations of *I. pumilio*. As pesticide we chose the organophosphate chlorpyrifos (CPF). CPF is one of the most frequently used pesticides worldwide (Eaton et al., 2008), situated within the top ten chemicals having the highest risk to organisms in surface waters in the UK (Johnson et al., 2017) and considered a priority pollutant in the European Water Framework Directive (2000/60/EC, Ojciec, 2000). Moreover, the trait-based studies that identified the ‘pace-of-life’ (Baas and Kooijman, 2015) and body size (Rubach et al., 2012) as predictors of sensitivity to pesticides explicitly studied exposure to CPF.

To study the impact of a multiple pulse application scenario of CPF under warming we focused on two key life history traits for damselflies (Stoks and Córdoba-Aguilar, 2012), mortality and growth rate, and on heat tolerance. As a measure of heat tolerance we determined the critical thermal maximum (CT_{max}), the upper temperature where animals no longer show body movements or muscular spasms (Verberk and Bilton, 2013). We looked at physiological mechanisms associated with effects on CT_{max}, by quantifying the activity of pyruvate kinase (PK) and lactate dehydrogenase (LDH), two enzymes related to aerobic/anaerobic energy production, and the amount of lactate as a metabolite of anaerobic respiration (Verberk et al., 2016). To further identify physiological mechanisms linked to the toxicity of CPF we also scored the activity of acetylcholine esterase (AChE), condition measures, and parameters related to oxidative stress and damage.

2. Methods

2.1. Collecting and housing

For each species, three randomly chosen populations in the northern region of the species’ areas were sampled. For *I. elegans* this were the Danish population Ahl Hage (56°10′59.66″N, 10°39′1.69″E) and the southern Swedish populations Nöbelövs mosse (55°44′6.00″N, 13°9′10.00″E) and Eriksö (58°56′4.90″N, 17°39′21.50″E). For *I. pumilio* this were the Danish populations Aarhus (56°6′30.20″N, 10°12′12.13″E), Grindsted (55°45′54.00″N, 8°58′16.00″E) and Tversted (57°36′27.86″N, 10°14′3.66″E). All populations occurred at shallow lakes with a rich aquatic vegetation. To equalize and minimize exposure to pesticides, all populations were chosen in natural areas with no surrounding agriculture (Coors et al., 2009; Cothran et al., 2013).

End June – early July 2014 twelve to eighteen mated females per population were collected and placed individually in small plastic vials with wet filter paper for oviposition. Eggs were transferred to the laboratory in Belgium. Throughout the experiment, eggs were incubated and larvae were reared at one of two water temperatures (20 °C or 24 °C) and a photoperiod of L:D 14:10 h. The lower temperature (20 °C) reflects the mean summer water temperature of ponds occupied by *I. elegans* at

this latitude (De Block et al., 2013). The sister species, *I. pumilio*, often co-occurs with *I. elegans* (Allen et al., 2010), yet tends to prefer shallower, sunnier ponds that are warmer (Fox and Cham, 1994). Larvae were reared individually in cups (7.5 cm height, 3.5 cm diameter) filled with 100 mL aged tap water, because they are cannibalistic (De Block and Stoks, 2004). Cups were placed in temperature-controlled water baths and every two weeks redistributed among the four water baths per rearing temperature. Larvae were fed *Artemia* nauplii ad libitum (206 ± 10 nauplii per food portion, mean $\pm$ SE, $n = 41$ food portions) six days a week. Three days after moulting into the final instar, the pesticide exposure treatment started at the respective rearing temperature. This assured that all larvae had been acclimated to their experimental temperature (starting from the egg stage) before we tested effects of the pesticide, and were standardized for their 'physiological age'.

2.2. Experimental design

To test whether the effects of chlorpyrifos (CPF) exposure on life history, heat tolerance and physiology depend on rearing temperature and predictably differ between two closely species with different life history strategies, we ran a nested full factorial experiment with three populations per species $\times$ two species $\times$ two CPF treatments (absent and present) $\times$ two temperatures (20 °C and 24 °C). An overview of the experimental procedure is given in Fig. 1. While the temperature treatment started at egg hatching, the CPF exposure treatment started three days after larvae had moulted into the final instar and lasted for seven days. During the CPF exposure period we scored survival and growth rate. Afterwards, larvae were directly tested (hence heated) for their heat tolerance and associated physiology.

Given lower initial numbers of *I. pumilio* eggs and different larval survival rates between treatment groups (see Results section) we started between 19 and 54 *I. pumilio* larvae and between 43 and 105 *I. elegans* larvae per combination of CPF treatment $\times$ temperature (total of 457 exposed larvae). During the pre-exposure period technical failure of one water bath at 24 °C that contained many *I. pumilio* larvae resulted in only about 20 surviving *I. pumilio* larvae per pesticide treatment at 24 °C. All larvae from this water bath were removed from the study, hence none of these larvae were included in the experiment and statistical analyses. This resulted in exact sample sizes for growth rate to vary between 17 and 48 for *I. pumilio* and between 42 and 69 for *I. elegans*. For both species, ca. 20 surviving larvae per treatment combination was

tested directly after the CPF exposure period for heat tolerance (CT_{max}). The exact sample sizes for CT_{max} varied between 19 and 21, only for *I. pumilio* at 24 °C there were only 14 and 16 replicates, in the absence and in the presence of CPF, respectively. Afterwards, larvae were frozen at -80 °C for measuring physiological traits linked to energy regulation and anaerobic metabolism. Exact numbers of each response variable per treatment combination are given in Figs. 2 and 3.

An extra subset of larvae underwent the same experimental procedure but was not tested for heat tolerances and directly frozen after the pesticide exposure period (see Fig. 1). This extra subset was used to quantify baseline (without heating) levels of the same physiological traits related to energy regulation and anaerobic metabolism as well as an extra set of physiological traits (AChE, condition measures, measures of oxidative stress and damage). Because of the technical water bath failure, no *I. pumilio* larvae were available at 24 °C for this extra subset. Therefore, we limited this test of baseline levels to the larvae of both species reared at 20 °C in the absence or presence of the pesticide. Given the incomplete design for this extra subset of larvae, the associated methods, results and discussion are presented as supplementary material in appendix S1.

2.3. Pesticide treatment

Larvae were exposed individually to four sequential pulses of chlorpyrifos (CPF), one pulse every other day, during the 7-day exposure period. The day after the larvae moulted into the final instar, the water in the cup was replaced by 100 mL of aerated, aged dechlorinated tap water. Three days later, larvae entered the 7-day CPF-exposure period. During the exposure period larvae received the same daily portion of *Artemia* as during the pre-exposure period but now seven days a week to ensure ad libitum food conditions during CPF exposure.

A pulse concentration of $1 \mu\text{g} \cdot \text{L}^{-1}$ CPF was chosen since a 6-day static renewal exposure to $1.5 \mu\text{g} \cdot \text{L}^{-1}$ CPF caused sublethal effects (e.g. a decrease in growth rate) in *I. elegans* larvae (Dinh Van et al., 2014), while current experiment allowed accumulation of the pesticide in the water. This pulse concentration is high, but realistic in edge-of-field water bodies where both species can be very abundant (Dijkstra, 2006) and where peak concentrations can exceed $100 \mu\text{g} \cdot \text{L}^{-1}$ due to runoff (Moore et al., 2002; Mazanti et al., 2003; Bernabò et al., 2011). In contrast with most studies that are investigating the impact of pesticides under global warming the here applied multiple pulse exposure regime does not

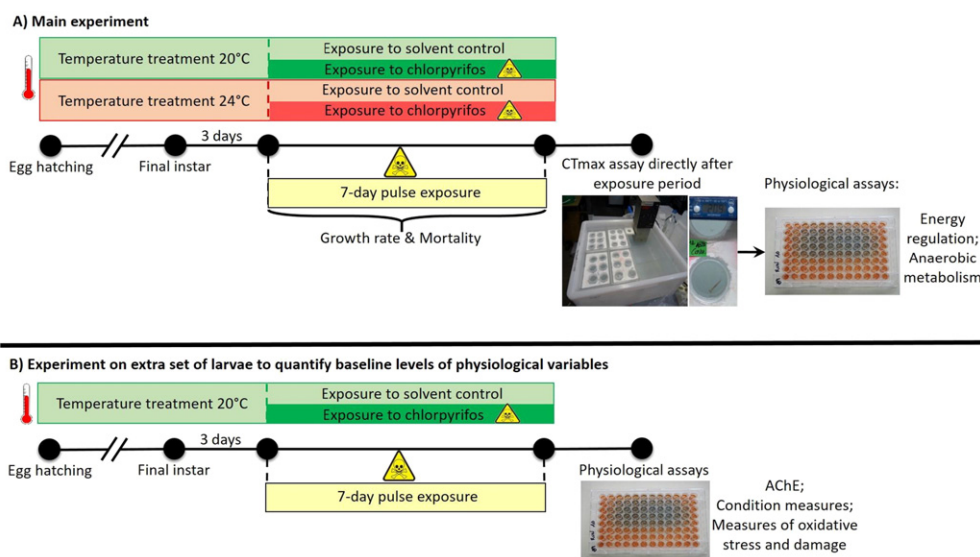


Fig. 1. Overview of the experimental procedure of (A) the main experiment and (B) the experiment on the extra set of larvae at 20 °C that underwent the same experimental procedure. This extra set of larvae was not tested for heat tolerances and directly frozen after the pesticide exposure period to quantify baseline levels of physiological variables. All larvae in (A) and (B) were reared and exposed individually. Results of (B) are reported in Appendix S1.

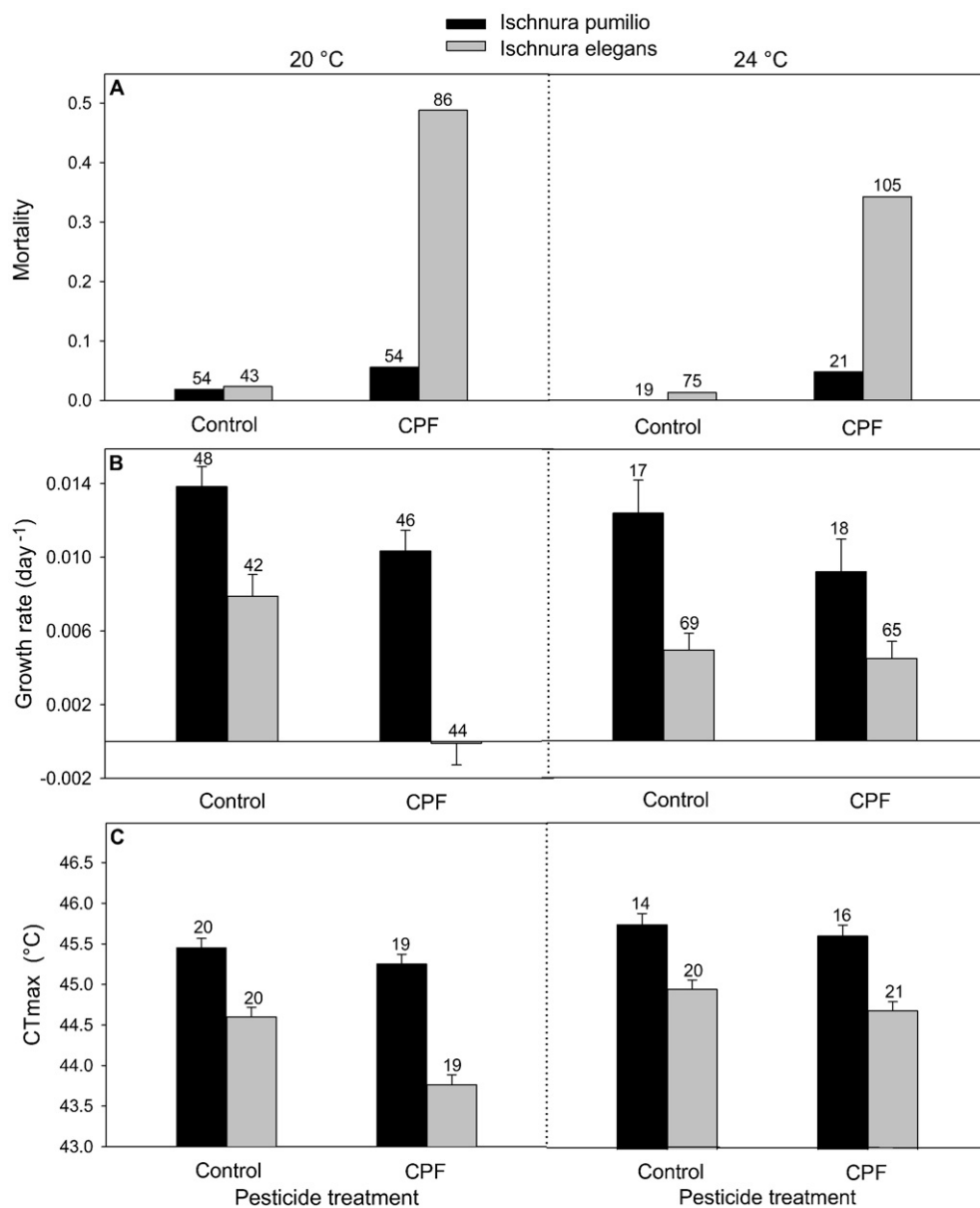


Fig. 2. (A) Mortality, (B) growth rate, and (C) heat tolerance (CTmax) of *Ischnura pumilio* and *Ischnura elegans* damselfly larvae as a function of chlorpyrifos (CPF) exposure and rearing temperature. For growth rate and CTmax means are given with 1 SE. The numbers above the bars represent sample sizes.

keep pesticide concentrations constant (via daily renewal of the medium) but allows potential accumulation after multiple pulses. This is a realistic scenario in edge-of-field ponds. Note that in this approach the resulting exposure concentration of the pesticide is likely to differ between temperatures (expected to be lower at 24 °C than at 20 °C, see Results section), yet because the same multiple pulse application treatment is used at both temperatures, it allows direct evaluation of the impact of this pulse scenario between temperatures. In other words, we are not testing the effect of a given CPF concentration between 20 °C and 24 °C, but the effect of a realistic CPF pulse application scenario between 20 °C and 24 °C.

The initial CPF concentration after the first pulse and the accumulated concentrations at the end of the 7-day exposure period at 20 °C and 24 °C (all based on a pooled sample of the medium of 20 cups) were measured using gas chromatography in combination with mass spectrometry by the research laboratory Lovap NV (Geel, Belgium). To achieve a pulse concentration of 1 µg·L⁻¹, a 200 µg·L⁻¹ stock solution was prepared by dissolving chlorpyrifos powder (Sigma-Aldrich, purity >99%) in absolute ethanol (100%). This stock solution was made in an amber glass

bottle, stored in the dark at 4 °C and renewed monthly. The stock solution was diluted with Milli-Q water to obtain a concentration of 0.02 µg·mL⁻¹. Five milliliters of the latter solution was added to 95 mL of dechlorinated tap water to obtain the pulse concentration of 1 µg·L⁻¹. In the solvent control treatment, we added the same amount of ethanol concentration (5 µL·L⁻¹). During the experiment the following physico-chemical parameters of the used water were measured in triplicate: conductivity, pH, dissolved oxygen and hardness.

2.4. Life-history variables

Mortality was checked daily during the 7-day exposure period. To obtain estimates of body size and growth rate, two species' traits related to sensitivity to chlorpyrifos (Congdon et al., 2001; Rubach et al., 2012), each larva was weighed to the nearest 0.01 mg using an electronic balance (AB135-S, Mettler Toledo®, Ohio, USA) at the start and at the end of the 7-day exposure period. Before weighing larvae were gently blotted dry with tissue paper, this gives reliable wet mass estimates that strongly correlate with dry mass (Stoks et al., 2005). The growth rate of each larva

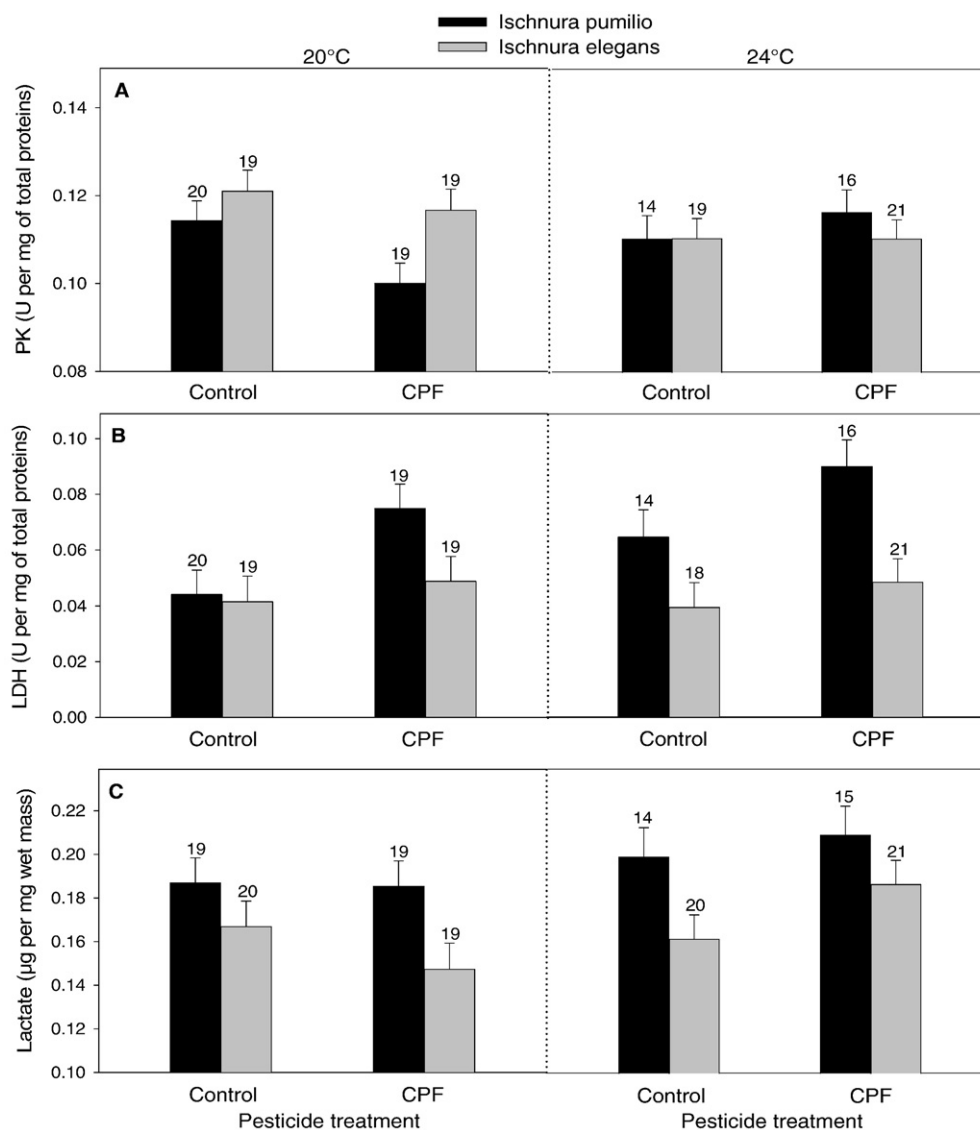


Fig. 3. Physiological traits related to aerobic/anaerobic metabolism of *Ischnura pumilio* and *Ischnura elegans* damselfly larvae as a function of chlorpyrifos (CPF) exposure and rearing temperature; activity levels of (A) pyruvate kinase (PK) and (B) lactate dehydrogenase (LDH), and (C) levels of lactate. Means are given with 1 SE. The numbers above the bars represent sample sizes.

was calculated as $[\ln(\text{final mass}) - \ln(\text{initial mass})] / 7 \text{ days}$ (Stoks et al., 2012).

2.5. Heat tolerance: CTmax

We estimated the heat tolerance by quantifying the critical thermal maximum (CTmax) directly after the 7-day exposure period. CTmax is considered a good proxy for the vulnerability of an organism to climate warming (Huey et al., 2012). We followed the dynamic method in which animals are heated at a constant rate until they reach a temperature (CTmax) where they no longer show any body movements or muscular spasms (Lutterschmidt and Hutchison, 1997; Verberk and Bilton, 2013). For the test, larvae were placed individually in open plastic vials (50 mL) filled with aged tap water (no pesticides present), which were floating in a temperature controlled aquarium. The water temperature in the test was initially 20 °C, and was then raised at a constant rate of 0.30 °C per minute using an aquarium heater. Larvae reared at 24 °C were added to the experimental vials when the water temperature reached 24 °C. The applied rate of temperature increase (ramping rate) is considered fast enough to avoid acclimation, but is also slow enough so that body temperatures of the test larvae can closely track the

increasing water temperature (Dallas and Rivers-Moore, 2012), and lies in the range commonly used to assess thermal tolerance of aquatic insects (e.g. Verberk and Bilton, 2011; Dallas and Rivers-Moore, 2012). The percentage saturation of dissolved oxygen in the water remained above 80% during all trials. After reaching the CTmax, larvae were immediately removed from the experimental vials and transferred to their rearing temperature in their original cups to record subsequent survival. Larvae were given ca. 20 min to recover from the heating before being weighed and frozen at −80 °C. The very few larvae (2 out of 150) that did not recover from the test were excluded from the analysis of CTmax.

2.6. Physiological variables

On the total body homogenates of the animals that had been exposed to the CTmax test we measured three variables related to energy production and anaerobic respiration: the activities of two important enzymes in the glycolysis, lactate dehydrogenase [LDH] and pyruvate kinase [PK], and the amount of the anaerobic metabolite lactate. Additionally, we measured protein content in the body homogenates using the

Bradford method to express specific enzyme activities as U per mg of total proteins. Detailed protocols for the assays are given in appendix S1.

During the rate-limiting final step of glycolysis, PK catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), yielding one molecule of pyruvate and one molecule of ATP (Voet and Voet, 1995). Pyruvate either enters the Krebs cycle for further production of ATP under aerobic conditions, or is reduced under anaerobic conditions by LDH to lactate when oxygen has been depleted (Voet and Voet, 1995). At thermal maxima, oxygen limitation is associated with the onset of anaerobic metabolism leading to the production of anaerobic metabolites, such as lactate (Verberk et al., 2013), likely resulting from upregulation of LDH. The excess in lactate will afterwards be reconverted by LDH to pyruvate to recreate glucose via gluconeogenesis in order to avoid acidification of the intracellular environment (Klowden, 2002).

The activity of lactate dehydrogenase (LDH) and pyruvate kinase (PK) were measured based on a modified protocol of McPeck (1999) and the total amount of lactate was measured based on Figenschou and Marais (1991). The amount of lactate was calculated based on a standard curve of commercial lactate and expressed as μg per mg wet mass. One unit LDH activity was defined as the amount of enzyme needed to catalyze the conversion of lactate into pyruvate to generate 1.0 mol of NADH per minute. One unit of PK was defined as the amount of enzyme needed to convert 1 μmol phosphoenolpyruvate to pyruvate per minute. Both LDH and PK activity were expressed as unit (U) per mg of total proteins. Measurements for all three variables were run in duplicate per larva and the means were used for statistical analyses.

2.7. Statistical analyses

We tested for effects of the pesticide treatment, rearing temperature and species, and their interactions on the different response variables using a set of linear mixed models. We added population nested within species to each model as a random effect. The nested factor population was, however, never significant (all $p > 0.090$), indicating consistency across populations of the same species.

Given that mortality is a binary response variable, we analysed it using generalized linear mixed models with a binomial error distribution and the logit-link function. Because mortality was near zero for all treatment combinations in the absence of the pesticide we ran a separate model to test for an effect of species and temperature in the subset of pesticide-exposed animals. We analysed growth rate, CTmax and all physiological variables using linear mixed models with a normal error structure and the identity link. For CTmax the larval mass was included as a covariate as the mass of aquatic organisms may be linked with their thermal sensitivity (Kelly et al., 2014). However, this covariate was not significant and therefore removed from the final model. When we detected significant interactions, we used linear contrasts to analyse differences between treatment groups; these were corrected for the false discovery rate using the method of Benjamini and Hochberg (1995).

All analyses were performed with the R software version 3.1.2 for Windows (R Core Team, 2014) using the 'lme4' package for constructing linear mixed models (Bates et al., 2012). To compute Wald chi-square statistics and p -values for fixed effects we used the package 'car' (Fox and Weisberg, 2011).

3. Results

3.1. Chlorpyrifos concentrations and physicochemical properties of the water

The initial CPF concentration after the first pulse was $1.00 \mu\text{g} \cdot \text{L}^{-1}$. The accumulated concentrations at the end of the 7-day exposure period were about twice higher at 20°C ($4.2 \mu\text{g} \cdot \text{L}^{-1}$) than at 24°C ($2.2 \mu\text{g} \cdot \text{L}^{-1}$).

The physicochemical properties of the water (mean $\pm$ 1 SD, $n = 3$ samples) were the following: conductivity: $766 \pm 1 \mu\text{S} \cdot \text{cm}^{-1}$, pH: 8.27

± 0.05 , dissolved oxygen: $8.53 \pm 0.03 \text{ mg} \cdot \text{L}^{-1}$ and hardness: $169.33 \pm 1.15 \text{ mg} \cdot \text{L}^{-1} \text{ CaCO}_3$.

3.2. Life history

In the absence of CPF nearly all larvae survived, irrespective of rearing temperature and species (Fig. 2A). In contrast, exposure to CPF caused mortality that strongly depended upon temperature and species (Fig. 2A). The analysis for only the CPF-exposed larvae showed that mortality was ca. 40% higher at 20°C compared to 24°C (Temperature, $\chi^2_1 = 4.11$, $p = 0.043$), and that mortality was ca. $8 \times$ lower in *I. pumilio* (ca. 5%) than *I. elegans* (ca. 42%, Species, $\chi^2_1 = 19.44$, $p < 0.001$). The temperature-dependent mortality in the presence of CPF did not differ between species (Temperature $\times$ Species, $\chi^2_1 = 0.13$, $p = 0.72$).

Larval growth rates were higher in *I. pumilio* than in *I. elegans* (Table 1, Fig. 2B). CPF reduced the larval growth rate (Table 1), yet this reduction depended on the rearing temperature and species (CPF $\times$ Temperature $\times$ Species, Table 1). Separate analyses per species revealed that for *I. pumilio* CPF caused a ca. 34% growth reduction across temperatures (Pesticide: $\chi^2_1 = 4.56$, $p = 0.033$; CPF $\times$ Temperature, $\chi^2_1 = 0.0093$, $p = 0.92$), while for *I. elegans* the CPF-induced growth reduction was strong (ca. 100%) at 20°C ($p < 0.001$) and not present at 24°C ($p = 0.70$) (CPF $\times$ Temperature, $\chi^2_1 = 15.16$, $p < 0.001$).

I. pumilio was smaller than *I. elegans* ($\chi^2_1 = 186.08$, $p < 0.001$), and this was more pronounced at 20°C than at 24°C (Temperature $\times$ Species, $\chi^2_1 = 7.89$, $p = 0.0050$). Indeed the mean ($\pm$ 1 SE) body mass at 20°C was 22.11 ± 0.58 for *I. pumilio* and 31.41 ± 0.57 for *I. elegans*; and at 24°C the body mass was 23.48 ± 0.66 for *I. pumilio* and 31.12 ± 0.55 for *I. elegans*.

3.3. Heat tolerance (CTmax)

I. pumilio had higher CTmax values than *I. elegans* (Table 1, Fig. 2C). CPF exposure reduced CTmax (Table 1), but this was not consistent across species and temperatures. The CPF-induced reduction in CTmax was present in *I. elegans* but not in *I. pumilio* (CPF $\times$ Species, Table 1, CPF effect in *I. pumilio*: $p = 0.17$, CPF effect in *I. elegans*: $p < 0.001$). Further, CPF reduced the CTmax only at 20°C but not at 24°C (CPF $\times$ Temperature, Table 1, CPF effect at 20°C : $p < 0.001$, CPF effect at 24°C : $p = 0.093$). However, as suggested by the three-way interaction (Table 1) and Fig. 2C, follow up analyses per species revealed that this CPF $\times$ Temperature interaction was only present in *I. elegans* ($\chi^2_1 = 4.97$, $p = 0.026$) and not in *I. pumilio* ($\chi^2_1 = 0.10$, $p = 0.75$). Higher rearing temperatures resulted in higher CTmax values (Table 1, Fig. 2C); this acclimation effect was stronger in *I. elegans* than in *I. pumilio* (Temperature $\times$ Species, Table 1).

3.4. Energy-related physiological traits after the CTmax test

I. pumilio had a lower PK activity than *I. elegans* at 20°C ($p = 0.016$) but not at 24°C ($p = 0.53$) (Temperature $\times$ Species, Table 1, Fig. 3A). The activity of PK is moderately significantly correlated with the CPF $\times$ Temperature interaction ($p = 0.069$, Table 1): CPF exposure tended to lower the PK activity at 20°C ($p = 0.070$) while this was not the case at 24°C ($p = 0.53$, Fig. 3A).

I. pumilio had a higher LDH activity than *I. elegans* (Table 1, Fig. 3B), but this seemed only the case at 24°C (Temperature $\times$ Species, Table 1, species effect at 24°C : $p < 0.001$, species effect at 20°C : $p = 0.10$). Exposure to CPF increased the LDH activity (Table 1, Fig. 3B), yet only in *I. pumilio* ($p < 0.001$) and not in *I. elegans* ($p = 0.28$) (Pesticide $\times$ Species, Table 1).

I. pumilio accumulated a higher amount of lactate than *I. elegans* ($\chi^2_1 = 13.16$, $p < 0.001$). A higher rearing temperature resulted in a higher amount of lactate ($\chi^2_1 = 4.52$, $p = 0.033$, Fig. 3E–F), but as indicated by the nearly significant Temperature $\times$ Pesticide interaction ($\chi^2_1 = 3.39$, $p = 0.065$) this temperature effect was only present for larvae

Table 1

Results of the general linear models testing for the effects of chlorpyrifos (CPF), rearing temperature and species on growth rate, CTmax, the activity of pyruvate kinase and lactate dehydrogenase, and the level of lactate of the damselflies *Ischnura pumilio* and *I. elegans*.

Effect	Growth rate		CTmax		Pyruvate kinase		Lactate dehydrogenase		Lactate	
	χ^2_1	P	χ^2_1	P	χ^2_1	P	χ^2_1	P	χ^2_1	P
CPF	25.30	<0.001	20.81	<0.001	1.35	0.24	10.68	0.0011	0.17	0.68
Temperature (Temp)	4.11	0.043	36.79	<0.001	0.22	0.64	1.95	0.16	4.52	0.033
Species (Spec)	14.77	<0.001	154.73	<0.001	2.11	0.15	14.08	<0.001	13.16	<0.001
CPF × Temp	13.69	<0.001	4.27	0.039	3.32	0.069	0.020	0.89	3.39	0.065
CPF × Spec	4.17	0.042	5.94	0.015	0.14	0.71	3.44	0.064	0.025	0.88
Temp × Spec	0.35	0.56	3.84	0.050	5.30	0.021	2.93	0.087	0.0027	0.96
CPF × Temp × Spec	4.13	0.042	2.43	0.12	1.57	0.21	0.11	0.74	1.05	0.31

exposed to CPF ($p = 0.012$) and not for larvae from the control condition ($p = 0.79$).

4. Discussion

The multiple pulse exposure to CPF affected all response variables, and these effects (except for lactate) differed between both species. *I. pumilio* showed the expected higher growth rate, confirming its faster 'pace of life', which is associated with the higher time constraints it encounters in ponds with a short hydroperiod (Debecker and Stoks, unpublished data). Yet, unexpectedly *I. pumilio* larvae were less vulnerable to CPF than *I. elegans* larvae. This sensitivity difference between both species contrasts with what was expected based on energy-allocation theory (Congdon et al., 2001) and empirical across-species trait-based sensitivity patterns for CPF (Rubach et al., 2012; Baas and Kooijman, 2015), yet can be explained by differences in physiology. This translated in the 'contaminant-induced climate change sensitivity' concept only being upheld in *I. elegans* and not in *I. pumilio*. We will focus on the CPF effects on *I. pumilio* and how these differed from the effects on *I. elegans*.

While our study provides information on how the impact of the pesticide may change under global warming, two important limitations should be noted. First, by rearing the animals at 20 °C and under simulated warming at 24 °C for one generation we only considered thermal plasticity in response to global warming, while animals may also show gradual thermal evolution (Stoks et al., 2014). Thermal evolution may reduce the impact of warming on the sensitivity to pollutants as we have shown for CPF-induced mortality in the here studied northern *I. elegans* populations (Op de Beeck et al., 2017a). Yet, this may not hold for all traits and gradual thermal evolution is not expected to counteract the negative effect of the pesticide on thermal tolerance in *I. elegans* (Op de Beeck et al., 2017b). Similar studies are needed on *I. pumilio* to assess the potential of gradual thermal evolution mitigating the here detected effects of the multiple pulse scenario of chlorpyrifos under warming. Second, we kept the temperatures constant during our experiment, while under natural conditions, diurnal temperature fluctuations may occur that may also shape the sensitivity to pollutants (Willming et al., 2013).

4.1. Accumulation of chlorpyrifos differs among temperatures

At the end of the 7-day exposure period, chlorpyrifos accumulated more at 20 °C than at 24 °C, which resulted in an almost twice as high end concentration at 20 °C compared to 24 °C. This reflects the well-known higher breakdown rates of organophosphorus pesticides at higher temperatures (Lartiges and Garrigues, 1995; Hooper et al., 2013; Noyes and Lema, 2015), leading to higher accumulated concentrations at the low temperature after repeated pulse applications without renewal of the medium. Multiple pulse exposure scenarios for pesticides such as CPF are realistic as these can be applied multiple times during the crop growth season (Van Drooge et al., 2001) and can enter pond systems on several occasions via runoff after rain or storm events (Dabrowski et al., 2002).

4.2. Effects of chlorpyrifos on life history

I. pumilio larvae showed minor (ca. 5%) mortality when exposed to CPF which contrasted with the high (ca. 42%) mortality in *I. elegans* larvae. The CPF-induced growth reduction (at 20 °C) was considerable (ca. 34%) in *I. pumilio*, yet much stronger (ca. 100%) in *I. elegans*. This lower sensitivity to CPF of *I. pumilio* matches its ability to occupy polluted manmade sites that are not suitable for most other damselflies (Allen et al., 2010). This finding contrasts with the expectation that more fast-lived species should be more sensitive to stressors (Réale et al., 2010). Some evidence for this expected pattern was found in *I. elegans* where larvae from faster growing populations suffered a higher reduction in growth rate after pesticide exposure (Dinh Van et al., 2014; but see Dinh Van et al., 2016). Moreover, building on the dynamic energy model it was recently shown that distantly related species with a higher metabolic rate (a key driver of a faster 'pace of life', Réale et al., 2010) are more sensitive to pesticides, including CPF (Baas and Kooijman, 2015). The expectation of a higher sensitivity to pollutants in more fast-lived species is based on energy mediated trade-offs predicting that animals investing more energy in growth and development, will as a consequence invest less in detoxification and damage repair (Congdon et al., 2001). A higher resting metabolic rate has been observed in *I. pumilio* (Debecker and Stoks, unpublished data), and the here observed higher activity of LDH (and higher amount of lactate, one of the end metabolites of anaerobic respiration, Verberk et al., 2013) further supports this (yet, PK activity was lower in *I. pumilio* at 20 °C). That the smaller *I. pumilio* is less sensitive than the larger *I. elegans* also contrasts with the general pattern that smaller aquatic invertebrate species are more sensitive to CPF (Rubach et al., 2012).

The physiological analyses on the extra subset of larvae (see Appendix S1) suggested some mechanisms that may contribute to the lower sensitivity to CPF of *I. pumilio* compared to *I. elegans*. Firstly, the activity of acetylcholinesterase (AChE), the target enzyme inhibited by CPF (Domingues et al., 2010), was higher in *I. pumilio*. Higher baseline activities of AChE have been linked to lower sensitivity to chlorpyrifos across instars in the midge *Chironomus riparius* (Buchwalter et al., 2004). Secondly, the baseline activities of the important anti-oxidant enzyme superoxide dismutase and the detoxifying enzyme glutathione-S-transferase were higher in *I. pumilio* suggesting a better protection against CPF effects mediated through oxidative stress. Thirdly, the fat content was also higher in *I. pumilio*, reflecting more energy reserves that can be used for detoxification and repair. In addition, given its higher metabolic rate (Debecker and Stoks, unpublished data), *I. pumilio* may have a higher elimination rate (k_{out}) for CPF, an important predictor for lower intrinsic sensitivity to CPF among aquatic invertebrate species (Rubach et al., 2012). Furthermore, Rubach et al. (2011) suggested that species with higher growth rates (such as *I. pumilio*) show less bioaccumulation of pesticides via the process of growth dilution. A final unexplored possibility is that *I. pumilio* may have a lower activity of Cytochrome P450 Monooxygenase (CYP450) which is associated with a lower neurotoxicity (e.g. a higher AChE activity, Dzul-Caamal et al., 2014). Differences in internal bioactivation of CPF to the more toxic

and reactive CPF oxon by CYP450 may explain species differences in sensitivity to CPF (Dzul-Caamal et al., 2014).

In apparent contrast with the general belief that organophosphate pesticides become more lethal at higher temperatures (Noyes et al., 2009; Noyes and Lema, 2015), the impact of CPF on *I. pumilio* was similar and, if anything, slightly higher at 20 °C. Indeed, the CPF-induced mortality and growth reduction were the same at both temperatures in *I. pumilio*. The absence of a stronger impact of the applied multiple pulse scenario of CPF at 24 °C can be explained by the higher degradation of CPF at 24 °C and the resulting twice lower CPF accumulation at 24 °C than at 20 °C in the medium. The higher degradation under warming thereby overruled the higher toxicity of CPF under warming (for *I. elegans*: Dinh Van et al., 2014). There is a weak signal that *I. pumilio* suffered more from CPF exposure at 20 °C given that only at 20 °C CPF exposure caused a lowered PK activity; this will result in lowered amounts of pyruvate and thus a decrease in energy status (Brooks and Storey, 1997). Notably, in *I. elegans* the higher CPF accumulation in the medium at 20 °C generated a stronger impact of CPF at the lower temperature both in terms of mortality and growth rate. This matches the overall higher sensitivity to CPF of *I. elegans*. Combining the patterns of both species indicates that higher breakdown rates of organophosphate pesticides (for example, chlorpyrifos) at higher temperatures (Lartiges and Garrigues, 1995; Hooper et al., 2013) may offset (in the less sensitive *I. pumilio*) and even overrule (in the more sensitive *I. elegans*) the higher toxicity of these pesticides at higher temperatures (Noyes et al., 2009; Holmstrup et al., 2010; Noyes and Lema, 2015, for the study species: Dinh Van et al., 2014). This suggests that under a realistic multiple pulse exposure scenario global warming can temper the impact of organophosphates, despite these being more toxic at higher temperatures (Noyes et al., 2009). Since previous studies used either a single pesticide application or kept concentrations constant under static renewal exposure, they did not allow accumulation of the pesticide in the medium after multiple pulse application. These contrasting outcomes on the impact of pesticides under different temperatures indicate the importance of considering realistic exposure conditions in determining pesticide impact under more natural settings, especially under future global warming scenarios.

4.3. Effects of chlorpyrifos on heat tolerance

Multiple pulse exposure to CPF did not result in a lower CTmax value in *I. pumilio*, whereas it did in *I. elegans*. This again reflects the lower sensitivity of *I. pumilio* to CPF compared to *I. elegans*. A reduced upper thermal tolerance after toxicant exposure is expected in general according to the ‘toxicant-induced climate sensitivity’ concept (Moe et al., 2013; Noyes and Lema, 2015). This concept has wide support in many taxa (e.g. Heath et al., 1994; Messaad et al., 2000; Patra et al., 2007; Bagwe et al., 2015). Likely, the accumulated CPF concentrations at the end of the exposure period were not high enough to detect sublethal effects on a higher order trait like CTmax in *I. pumilio* (yet we did detect a sublethal effect on growth rate). Furthermore, *I. pumilio* experiences strong selection for a higher thermal tolerance as it often occurs in shallow, temporary ponds (Allen et al., 2010) where diurnal temperature fluctuations (Kern et al., 2015) and the impact of heat waves are much stronger. In line with this, in a comparison of three frog species, tadpoles of the species that experienced more daily temperature fluctuations had a higher thermal tolerance to reduce damage caused by temperature extremes (Kern et al., 2015). This is supported by the overall higher CTmax value of *I. pumilio* compared to *I. elegans*. The already high CTmax value at 20 °C may also explain that the increase in CTmax after thermal acclimation to higher temperatures (a general response in ectotherms, Rajaguru, 2002; Dallas and Rivers-Moore, 2012; Gunderson and Stillman, 2015) was less strong in *I. pumilio* than in *I. elegans*. Whatever the underlying reason, this illustrates that pesticide run-off followed by a heat wave may change the competitive interactions between both species in favor of the otherwise competitive inferior *I. pumilio*.

The underlying rationale for the ‘toxicant-induced climate sensitivity’ concept is a reduction in aerobic scope (Moe et al., 2013; Noyes and Lema, 2015). Under increasing temperatures (as imposed in the CTmax test) animals will increase their resting metabolic rate much more than their maximum metabolic rate resulting in a lower aerobic scope (Verberk et al., 2016). To deal with the associated mismatch between oxygen demand and supply, hence energy deficiency, animals often rely on energy production through anaerobic metabolism (Pörtner et al., 2007; Verberk et al., 2016). When organisms experience exposure to contaminants, more energy needs to be invested in maintenance costs for detoxification and damage repair (Sokolova and Lannig, 2008), thereby also increasing resting metabolic rate, hence further reducing the aerobic scope. Exposure to CPF indeed leads to high energetic costs and can affect aerobic performance (Narváez et al., 2016). This was suggested in our results by the increased activity of lactate dehydrogenase (LDH) when larvae had been exposed to CPF (also in larvae that were not heated, appendix S1). Note that the increased LDH activity after pesticide exposure did not result in higher levels of lactate; possibly lactate was rapidly reconverted to pyruvate to avoid acidification of the intracellular environment (Klowden, 2002). While the observed increase in LDH activity under CPF exposure suggests a limitation in aerobic scope (Verberk et al., 2016), this did not translate into a lower CTmax in *I. pumilio*. Changes in metabolic enzyme activities do, however, not always reflect changes in metabolic rates (Kern et al., 2015), and therefore might not always up-scale to higher order traits (Seebacher et al., 2010) such as CTmax.

5. Conclusions

The here reported strong differences in sensitivity to CPF in the closely related species challenge both the phylogeny-based approach and the trait-based approach. Our results urge caution when extrapolating pesticide sensitivities toward congeneric species with unknown sensitivity (as e.g. done in the SPEAR index, Liess and von der Ohe, 2005). Our findings also challenge the hypothesis that faster growing and smaller species should be more vulnerable to pollutants (Rubach et al., 2012; Baas and Kooijman, 2015). This indicates that currently used traits related to metabolism and body size, which have been successful in estimating sensitivity to chemicals in trait-based approaches when comparing distantly related species (Rubach et al., 2012; Baas and Kooijman, 2015), might not allow to correctly estimate sensitivity of closely related species. Our results suggest that trait-based approaches can be strengthened by integrating physiological traits related to detoxification, oxidative stress and the target mechanism of pesticides (see also Rico and Van den Brink, 2015), traits that are largely ignored in trait-based approaches (Poteat et al., 2015).

A key observation with regard to ERA in a warming world was that the here observed differences in sensitivity to pesticides in the closely related *Ischnura* species also resulted in differential support for the two interaction pathways at the interface of ecotoxicology and global change biology. Indeed, while *I. elegans* followed both the concepts of “toxicant-induced climate sensitivity” (Noyes and Lema, 2015) and “climate-induced toxicant sensitivity” (yet opposite to general belief, Noyes and Lema, 2015), this was not the case for *I. pumilio* although the used CPF concentration did cause sublethal negative effects. This is an important finding as both principles play an important role in understanding and predicting the impact of pesticides in a warming world (Hooper et al., 2013; Noyes and Lema, 2015).

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Appendix A. Supplementary data

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